THE EFFECTS OF BIOCHEMICAL MUTATION ON THE VIRULENCE OF *BACTERIUM TYPHOSUM*: THE LOSS OF VIRULENCE OF CERTAIN MUTANTS.

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PREVIOUS papers (Bacon, Burrows and Yates, 1950*a* and *b*) reported the isolation and characterization of a number of biochemical mutants of *Bacterium typhosum* and recorded their virulence for mice. Whilst the majority of mutants remained unaltered in virulence, a number of specific mutations resulted in a marked decrease, the virulence of purine mutants in particular being of a very low order. The experiments reported here were designed to investigate possible reasons for the loss of virulence associated with these specific mutations.

MATERIALS AND METHODS.

Strains.

Five strains deficient in their ability to synthesize purines (479, 20080, 20092, 20095 and 20102), one (20063) requiring *p*-aminobenzoic acid (PABA), three (8505, 19341, 19662) requiring aspartic acid (" aspartic-less ") and three anomalous strains (9749, 20085, requiring histidine and 20058, requiring cystine) were investigated. Strains 479, 20063 and 8505 received most attention, as representatives of the three growth factor types with which marked loss of virulence was invariably associated.

Suspensions.

For convenience in obtaining the large numbers of relatively avirulent organisms required for lethal doses, cultures grown for 16 hours on tryptic meat digest agar (T.M.A.) were employed in place of cultures on growth factor supplemented minimal medium. In all other respects the details of preparation of suspensions, media and buffer and methods of conducting virulence tests are as previously described (Bacon, *et al.*, 1950*a* and *b*).

EXPERIMENTAL AND RESULTS.

In common with other purine-less mutants, 479, as previously noted, conforms with the accepted criteria defining virulent strains of *Bact. typhosum*. It grows readily on T.M.A., producing smooth, circular, more or less opaque colonies indistinguishable from those of the virulent strain Ty22 from which it was derived. It is morphologically identical with Ty22, and equally sensitive to lysis by specific Vi phage. Both mutant and parent are freely motile from growths on T.M.A. and non-motile when grown on minimal medium. They both resist O-aggluti-

nation in the living state, and possess Vi and O somatic antigens as determined by agglutination of living suspensions by Vi antisera, and of heat-killed suspensions by O antisera. No qualitative differences in their agglutinogenic structure were revealed by reciprocal absorption tests with their antisera. Quantitatively, 479 is slightly more O-agglutinable than the parent, suggesting a lower complement of Vi antigen, this being confirmed by comparative quantitative immunization experiments in rabbits using alcoholized vaccines (Felix, 1941). It seemed unlikely, however, that the quantitative difference in the abundance of Vi antigen in this mutant was responsible for the low virulence observed, in view of the fact that its O-agglutinability was of the same order or less than that of a number of other mutants of high virulence. Further, mutant 20080, which is as resistant as Ty22 to O-agglutination, is of the low virulence shown by 479, and mutants 479 and 20080 are of lower virulence than the classical strain 0901, which is completely devoid of Vi antigen. The toxicities of 479 and Ty22 suspensions killed by heating at 60° C. for 30 minutes are of the same order.

Comparative survival of mutant 479 *and Ty*22 *in mice.*

Although mutant 479 was known to be recoverable by culturing the spleens of mice seven days after injection with 500 million organisms, and therefore was able to survive in the host for this period, it was considered of interest to compare its survival with that of the virulent Ty22. The nutritional difference existing between the two strains permitted the enumeration of each in mixed suspensions, and allowed the survival rates of the two strains to be determined in the same animal, and therefore under identical environmental conditions. Mice were injected with a mixed suspension containing 1 million cells of 479 and 1 million of Ty22. At intervals of two days after injection pairs of mice were killed by cervical dislocation, their spleens removed, macerated in a blendor, serially diluted and plated on minimal medium supplemented with adenine to a final concentration of 2μM. On such a medium mutant 479 produced very small colonies readily differentiated from those of Ty22. The mutant in these experiments was found to be eliminated from the host considerably more rapidly than the parent. Zelle (1942), investigating the relative survival of colonially distinguishable strains of *Salmonella typhi murium*, similarly showed the rough avirulent strains to be less well suited to survival in the host than the virulent smooth strains.

We considered this possibly reflected a differential resistance to phagocytosis between 479 and Ty22. *In vitro* tests by the method of Bhatnagar (1935) using rabbit or mouse leucocytes showed the two strains to be phagocytosed equally and at a considerably lower rate than strain 0901.

Phagocytosis *in vivo* was investigated in the mouse as follows: Phagocytes were mobilized to the peritonea of a series of mice by the injection of doses of 1 ml. of 2 per cent (w/v) wheat starch in saline intraperitoneally. After 16 hours 500 million Ty22 or an equal number of 479 cells were injected by the same route, and pairs of mice sacrificed after 10, 15, 20, 30, 60, 120, 180 and 240 minutes. Films were prepared of the peritoneal exudate, stained (Leishman) and the phagocytic indices of the two strains determined. From 120 minutes to the termination of the experiments estimates of the numbers of phagocytes present in the peritoneum were made, using a haemacytometer, and after 4 hours

the numbers of free organisms were determined by plate counts of the peritoneal washings. The latter counts would include ingested organisms capable of producing colonies on agar.

Over the initial 2-hour period following injection the phagocytic indices of the two strains were similar. Subsequently the numbers of Ty22 ingested exceeded those of 479. It was very evident, however, from the films prepared that the background of uningested organisms in the Ty22 series progressively increased with time, whilst in the 479 series the converse held, so that after 4 hours the phagocytes themselves exceeded the numbers of uningested 479 cells. The injected dose of Ty22 had in this time increased to give 2230 million free organisms, and the same dose of 479 decreased to 13 million. The numbers of phagocytes with ingested organisms in the Ty22 series was then 26 million, and in the 479 series 45 million, the latter figure indicating that few, if any, ingested organisms were capable of producing colonies on agar. The loss of virulence of the mutant therefore appeared to result from its inability to outstrip phagocytosis by division, suggesting sensitivity to an inhibitory agent present in peritoneal fluid, ineffective against Ty22, or that the mutant was unable to multiply owing to the absence of specific growth factors from the environment. The peritoneal fluid was therefore tested for the presence of available growth factors for 479, and for a random selection of virulent and avirulent mutants.

Availability of specific growth factors in the mouse.

The peritoneal cavities of 30 mice were each rinsed thoroughly with 2 ml. citrated minimal medium using aseptic technique, the washings centrifuged free from cells and debris, incubated for sterility and pooled. To tubes of 6 ml. minimal medium, 1 ml. of washings was added and the tubes seeded with a small washed inoculum of the different mutants. Controls comprising minimal medium with and without specific growth factor and supplemented with both peritoneal fluid and growth factor were included for each mutant. Growth was recorded daily by plate counts or turbidimetrically.

The data obtained for mutant 479 and the virulent mutant 10020 requiring pantothenate are recorded in Table I, and the complete results summarized in Table II.

It is clear from Table II that the peritoneal fluid of normal mice contains an abundance of those growth factors required by mutants showing a high virulence. It is equally clear that those required by purine- or PABA-less mutants are of limited availability, and could well account for the low virulence shown by them. The rapid and abundant growth obtained in minimal medium supplemented with both peritoneal fluid and growth factor indicated the absence of any inhibitory substance, effective against mutants of low virulence, at the dilution of peritoneal fluid employed.

An examination of the distribution of viable organisms in mice moribund from an infection of Ty22 injected intraperitoneally showed the vast majority to be localized in the peritoneal cavity. Here the organisms were approx. 1000 times more abundant than the total in the blood stream, and considerably exceeded the sum of those recoverable from all other parts of the animal. It is reasonable to assume, therefore, in the absence of information to the contrary, that the vast numbers developing in the peritoneum are mainly responsible for the pathological

TABLE I.—*Determination of Available Growth Factors for Mutants in Mouse Peritoneal Fluid.*

Mutant.	Minimal medium supplemented with—	Growth: Organisms per ml. × 10^{-6}. Days' incubation. 0.	1.	3.	5.	Organisms supported by growth factor available per peritoneum × 10^{-6}.
479	..	0·003	0·004	0·0004	0	
	Adenine + aneurin	0·003	0·007	100	1550	
	Peritoneal fluid	0·002	0·002	0·0001	0	0
	Adenine + aneurin + peritoneal fluid	0·003	750	1350	1550	
10020	..	0·002	0·005	0·002	0	
	Pantothenate	0·004	0·015	820	1530	
	Peritoneal fluid	0·004	550	660	730	10,200
	Pantothenate + peritoneal fluid	0·003	900	1250	1530	

TABLE II.—*Numbers of Mutant Organisms Supported by the Available Growth Factors in the Peritoneal Fluid of a Mouse.*

	Mutant.	Growth factor.	Organisms × 10^{-6}.
Mutants of high virulence	8974	Nicotinamide	15,000
	20039	Aneurin	14,400
	20022	Phenylalanine	13,900
	10020	Pantothenate	10,200
	9355	Valine + isoleucine	8,400
	5459	Methionine	6,300
	20057	Cytosine	6,300
Mutants of lowered virulence	6346	Histidine	3,000
	20082	Leucine	1,800
	20034	Glycine	1,800
	8505	Aspartic acid	1,200
	20063	PABA	0·56
	20080	Purine	0·0
	479	Purine + aneurin	0·0

condition, and that a study of the conditions for growth in the peritoneum is pertinent to a study of typhoid infection in mice injected by the intraperitoneal route.

Recovery of virulence by injection of specific growth factors.

If the loss of virulence of purine- and PABA-less mutants is attributable to the limited availability of specific growth factor from the peritoneal exudates of the host, it should be possible to recover the virulence of the parent type by injecting

with the mutant a suitable quantity of its growth factor. The high toxicity of adenine to mice prevented the addition of more than 2·0 mg. of this purine. A dose of 0·5 mg. injected with 479 suspensions resulted in a two-fold increase in virulence. Prolongation of the effective time over which the growth factor was available by the injection of 0·17 mg. adenine with the organisms, followed by two similar doses at 2 and 4 hours later, resulted in a four-fold increase in virulence. The much lower toxicity of hypoxanthine permitted growth factor additions of at least 8 mg. per dose. Graded doses of this purine resulted in the enhancement of the virulence of 479 to a maximum equal to that shown by Ty22 in the presence of hypoxanthine, as illustrated in Fig. 1. Hypoxanthine alone

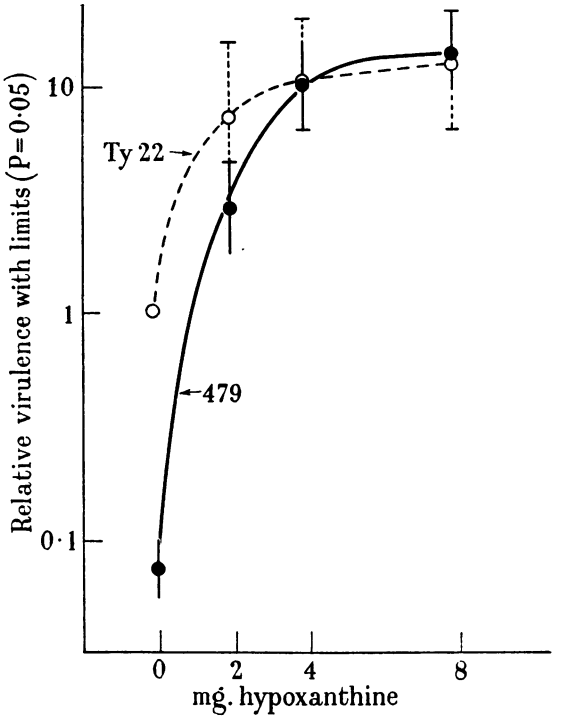

FIG. 1.

was without apparent effect on mice in doses of 16 mg. The low solubility of this purine in buffer necessitated its administration as a fine suspension, which probably enhanced the effect by retarding its elimination from the peritoneum.

In a similar manner the virulence of PABA-less mutant 20063 was raised to the level of Ty22 by the injection of 2 mg. PABA suspended in arachis oil. In experiments of this nature it is necessary to prolong the existence of the added growth factor in the peritoneum, until a lethal dose of organisms has had time to develop from the injected dose. With the progress of time the demands of the organisms for growth factor probably increase logarithmically, whilst the injected growth factor is being eliminated at an unknown, but probably rapid, rate. A considerable excess of growth factor must therefore be provided initially to ensure

its presence in the later stages of infection, or the factor must be injected with a vehicle (e.g. arachis oil) permitting its slow liberation during the infection. Doses of 2·5 mg. PABA dissolved in buffer had only a slight virulence-enhancing effect on mutant 20063, presumably owing to very rapid elimination from the peritoneum. The specificity of the injected growth factors in enhancing the virulence of purine and PABA-less mutants is illustrated in Table III.

TABLE III.—*Specificity of Injected Growth Factor in Enhancing Virulence of Purine-less and of PABA-less Mutants.*

Organism.	Deaths out of 20 mice injected with 4 mg.:—		
	—	hypoxanthine	PABA
—	0	0	0
Ty22	13	18	11
479	0	16	0
20063	0	1	16

Dose : 20 million organisms. Growth factors suspended in 0·25 ml. arachis oil.

For full growth in minimal medium aspartic-less mutants require the addition of L-aspartic acid to a level of 1000μM as compared with 40–100μM levels required by the majority of other amino-acid- or purine-requiring mutants. It is probable therefore that large amounts of growth factor would initially be necessary to ensure the presence of sufficient quantities in the later stage of infection of mice with aspartic-less mutants. Doses of 6 mg. of aspartic acid suspended in arachis oil, whilst having no effect on the virulence of Ty22, considerably enhanced the virulence of the aspartic-less mutant 8505, but did not result in full enhancement to the level shown by the non-exacting parent. Injection of larger amounts of aspartic acid or of calcium aspartate showed some toxicity to the test animals and could not be employed. Aspartic acid injected in doses of 6 mg. resulted in reduction of the average lethal dose of mutant 8505 from 122 millions to 29 millions.

Reversion of mutants to the non-exacting state and its effect on their virulence.

Washed inocula of mutants of low virulence were heavily seeded (approx. 2×10^8 cells per plate) on minimal agar and incubated. Cells reverting to the non-exacting condition are capable of growth on this medium, and recognizable as large colonies against the background film of mutant cells. Large colonies which developed were isolated and confirmed as typhoid organisms by phage testing, and for their ability to grow in minimal medium without growth factors. By this means we obtained reverted strains of 479, 20092, 20063, 19662, 9749 and 20058. We have not succeeded in obtaining similar reversions of mutants 20080, 20095, 20102, 19341, 8505 or 20085 despite repeated attempts at their isolation, involving the plating of over 2×10^9 organisms in several experiments. It is of interest to record that mutant 479 reverted to the loss of its requirement for a purine and for aneurin in a single step, and no back mutations to separate loss of one or other requirement have been isolated.

Table IV records the virulence of the reverted mutants obtained.

It is seen that biochemical mutants which have no accompanying morphological or colonial variation recovered a virulence comparable with that of the

TABLE IV.—*Change in Virulence of Mutants on Reversion from Growth Factor Dependence to Independence.*

Mutant.	Relative virulence with limits (Ty22 = 1·0, P = 0·05).	
	When dependent.	When independent.
479	0·04 (0·03–0·05)	0·70 (0·49–1·0)
20092	0·03 (0·01–0·04)	0·55 (0·33–0·88)
19662	0·14 (0·09–0·2)	0·70 (0·4–1·0)
20063	0·04 (0·03–0·06)	1·1 (·78–1·6)
9749	0·05 (0·03–0·07)	0·10 (0·06–0·16)
20058	0·02 (0·01–0·03)	0·06 (0·04–0·09)

parent on reversion to the non-exacting condition, suggesting that the biochemical mutation was responsible for the observed loss of virulence. Biochemical mutants 9749 and 20058 are also morphological variants. These retain their low virulence and their morphological variation when reverted to independence of added histidine or cystine respectively. The loss of virulence and morphological variation shown by these mutants evidently is not attributable to biochemical mutation.

Variation in virulence.

Throughout this work, conducted over a period of several months, it was found that the relative virulence with respect to Ty22 of mutants of lowered virulence was not constant. Virulence assays repeated at later dates did not confirm those made several months or even weeks previously. The average lethal dose of mutant 479, for example, in June was 3300 million organisms (relative virulence to Ty22 0·009). This number decreased with reasonable regularity to a minimum of 232 (relative virulence 0·09) by September, and subsequently increased. The average lethal dose of mutant 20063 varied erratically between extremes of 1025 million (relative virulence 0·015), and 176 million (relative virulence 0·10). In the light of the experiments reported in this paper it is suggested that the variation in virulence reflects a seasonal or otherwise variation in the composition of mouse peritoneal fluid. Support is given to this view by the fact that in a repeat assay of available purines in mouse peritoneal washings, at the time of highest virulence of mutant 479, this mutant was able to develop to a level of 210 million (cf. Table II), as opposed to no growth in the original assays made when 479 was at its lowest virulence.

The average lethal dose of the standard Ty22 also varied over the period of these experiments, particularly during the period May to July. In experiments conducted at 3–5-day intervals the average lethal dose of 13 million on May 3 became successively 16, 29, 40, 42, 60, to reach a maximum of 73 million on June 9, and subsequently declined 43, 33, 27 to 22 million by July 6.

Effect of dietary growth factors on virulence of mutants.

It was considered of interest to investigate whether, by incorporating specific growth factors in the diet of our experimental animals, we would be able to raise their availability in the host and thereby specifically increase the virulence of our

mutants. Mice maintained on a normal diet but fed 10 mg. hypoxanthine in their drinking-water daily for 4 days prior to challenge with mutant 479 showed no decrease in resistance to infection. Similar negative results were obtained with mice ingesting 20 mg. asparagine daily for the same time, and challenged with mutant 8505. However, it was possible considerably to enhance the virulence of mutant 20063 by the incorporation of small amounts of PABA in the diet, as illustrated in Table V. The results indicate a slight cumulative effect of dietary PABA on the available concentration of this growth factor in the mouse. The incorporation of 10 mg. PABA in the diet on 5, 3 and 1 days prior to challenge permitted mutant 20063 to show a virulence equal to that of Ty22.

TABLE V.—*Effect of Dietary PABA on the Virulence of Mutant* 20063.

Culture.	PABA given on days— 5.	4.	3.	2.	1.	Challenge. Dose × 10^{-6}.	Deaths.
20063	—	—	—	—	—	400	3
	—	—	—	—	—	100	0
	—	—	—	—	+	100	16
	—	—	+	+	+	100	18
	+	+	+	+	+	100	20
Ty22	—	—	—	—	—	40	12
	—	—	—	—	—	10	8
	—	—	—	—	+	10	9
	—	—	+	+	+	10	10
	+	+	+	+	+	10	3
—	+	+	+	+	+	—	0

20 mice per treatment, each given 2 ml. water or PABA solution (5 mg. per ml.) on the days prior to challenge as indicated. + = PABA; — = water.

Variation in the amount of PABA in the diet of our test animals (which until received by us was not within our control) could well explain the variation in virulence experienced with mutant 20063. It is probable that factors other than dietary sufficiency control the level of available purines in the mouse, since mammals are independent of an exogenous supply of these compounds (Benedict, 1917).

Synergistic action of mutants.

If the low virulence of purine- and PABA-requiring mutants is due to a limitation of available growth factor preventing their development to a level toxic to the host, when two mutants having different growth factor requirements are simultaneously injected each theoretically should grow to the limits of the available factors, and the total number of organisms developing should equal the sum of each separately. That is, a double infection with two such mutants would be expected to be more lethal than either separately. This was found to be the case, as illustrated in Table VI. Mutants of high virulence, e.g., the nicotinamide-less 20035 and phenylalanine-less 20022, injected together were no more virulent than either separately; neither were two low virulence mutants having the same growth factor requirement (mutants 479 and 20102).

TABLE VI.—*Synergistic Effect of Purine and PABA-less Mutants.*

Total dose of organisms $\times 10^{-6}$.	Deaths out of 20 mice injected with—		
	479.	20063.	479 + 20063.
100	0	0	2
200	0	1	17
400	2	1	18
800	9	10	20

Although the injected suspension of 479 plus 20063 contained approximately equal numbers of each mutant, and the virulence of these mutants was at the time of these experiments of the same order, about 30 times as many PABA- than purine-requiring organisms were recoverable from mice moribund from the double infection. Mutant 479, like Ty22, when grown in minimal medium plus growth factor liberates into the medium effectively large amounts of growth factor active for our PABA-requiring mutant. 20063 similarly grown, like Ty22, liberates effectively small amounts of growth factors supporting purine-requiring mutants. It is probable, therefore, that in a mixed infection as described above the PABA-less mutant obtains a considerable amount of its specific growth factor from over-production by mutant 479. Such an effect is readily demonstrable *in vitro* by growing mutants 479 and 20063 in media containing suboptimal amounts of PABA, purines and aneurin, and observing the total growth occurring in tubes inoculated with each mutant separately and with both together.

DISCUSSION.

Biochemical mutation to particular growth factor dependence in *Bact. typhosum* has clearly been shown to modify considerably the virulence of this organism. Those growth factors required by mutants showing a high virulence are abundant in mouse peritoneal fluid, whereas purines, *p*-aminobenzoic acid and aspartic acid are of limited availability. Following intraperitoneal inoculation, the vast majority of viable organisms in mice moribund from the infection are to be found in the peritoneal cavity. The addition of purines, PABA or aspartic acid to the peritoneum specifically enhances the virulence of mutants dependent on the presence of these factors for growth ; and the reversion of these mutants to independence of these factors results in full recovery of their virulence. These observations are consistent with the view that loss of virulence of purine- PABA- or aspartic acid-requiring mutants results from their inability to develop from a sublethal inoculum to a level fatal to the host because of insufficient availability of their specific growth factors in the host.

The low virulence of the morphologically altered mutants 9749 and 20058 is independent of their biochemical mutation to requirements for histidine or cystine. On reversion to the non-exacting state they retain their low virulence and morphological variation. These mutants are therefore not at variance with the statement, based on the evidence of 8 other histidine-less and 10 other cystine-less mutants, that mutation to these growth factor requirements *per se* has little effect on the virulence of mutants of these types.

With our *p*-aminobenzoic acid dependent mutant 20063 we have been able to give a clear demonstration of the effect of specific dietary constituents on the resistance of mice to infection with this mutant, and to afford an explanation for the variation in virulence of the strain observed over a period. The increased resistance to infection with avian malaria parasites shown by pantothenic acid- or riboflavin-deficient chicks in the experiments of Brackett, Waletsky and Baker (1946) and of Seeler and Ott (1944) by analogy may be indicative of the growth factor dependence of these protozoa on these vitamins.

Our growth factor feeding experiments imply that whereas the mouse finds it necessary to maintain a constant low level of available purines for any given set of environmental conditions, the level of available PABA is not so critical, and accumulation of this factor can readily be achieved. The physiological conditions governing the availability of purines are unknown. As judged by their effect on the virulence of purine-requiring mutants, dietary purines are apparently not absorbed, or are made unavailable to such mutants very rapidly after absorption. Experiments of this nature emphasize the importance of maintaining experimental animals under constant dietary and environmental conditions throughout the whole course of an investigation on a host-parasite relationship, particularly where the parasite under investigation is an exacting organism.

The variation in virulence shown by suspensions of Ty22 prepared by a standard method from dried cultures also could be explicable on a basis of available nutrilites in the host. If it is assumed that the primary defence mechanism against typhoid infection in mice is phagocytic, then factors enhancing the growth rate of the organism would allow it the more easily to outstrip phagocytosis by division. Presumably the growth rate is controlled by the synthetic process which proceeds at the slowest rate. In a medium containing the majority of metabolites the growth rate will be governed by the rate of synthesis of those essential metabolites which are not available in a preformed condition. In the mouse both purines and PABA have been shown to be limiting factors, so that for growth these substances must be synthesized by Ty22, and the rate of their synthesis will limit the growth rate of the organism. Provision of these factors would therefore be expected to enhance the growth rate up to a maximum until the synthesis of some other essential metabolite becomes limiting. Our experiments have shown that increasing additions of both purines and PABA to the peritoneum increase the virulence of Ty22 up to a maximum, as would be expected on the above hypothesis. Purines are particularly effective in this respect, giving a 12-fold virulence enhancement of Ty22 in experiments conducted in September, when the behaviour of mutant 479 indicated the level of available purines in the mouse to be at a maximum. The 3-fold enhancement of the virulence of Ty22 given by PABA whilst purines are of limited availability could be due to the inter-relationship between purine and PABA synthesis suggested by the work of Snell and Mitchell (1942) and others. It is not unlikely that a particular nutrilite in addition to purines and PABA became limiting over the period of decrease in virulence of Ty22 between May and June, and subsequently returned to normal availability from June to July.

A clear example of the synergistic effect of two mutants of lowered virulence has been given in our experiments with mutants 479 and 20063. The effect most probably is attributable to reciprocal growth factor excretion by the two strains, allowing the development of each to a higher level in the host than would other-

wise have been possible. Similar synergism whereby organisms derive nutritional advantage from each other may well occur in some natural infections.

Where no serological or morphological differences are demonstrable between strains of pathogenic organisms of high and low virulence, this study emphasizes the necessity of conducting a close examination of the nutritional requirements of such strains to exclude a nutritional basis for the differences in virulence. It would, for example, be desirable to examine the nutritional requirements of the pathogenic and apathogenic strains of *Salmonella typhi-murium* described by Maaloe (1948) to confirm that the loss of invasiveness of the apathogenic strain did not result from a nutritional limitation. It is possible that the host and strain specificity of pathogenic organisms is in some cases determined by the nutritional requirements of the organism, a fundamental necessity for the manifestation of virulence in an exacting pathogen being the presence of adequate supplies of its essential growth factors in the host it is attempting to parasitize. Seasonal variation in the incidence of particular infections may from a similar cause be dependent on a seasonal variation in the abundance of available nutrilites in the host.

The production of particular nutritionally deficient mutant strains of pathogenic organisms provides a means of obtaining potentially fully virulent strains (prevented from manifesting their virulence owing to the limited availability of a specific growth factor from the host) to facilitate studies on a particular host-parasite relationship. The use of such strains as living vaccines is theoretically of immunogenic value, although involving a number of practical difficulties. Not the least would be the prevention of reversion to the non-exacting condition. The rate of spontaneous reversion could, however, greatly be reduced by incorporating a multiple genetic blockage in the synthesis of that factor whose deficiency results in loss of virulence, or by incorporating into one mutant a number of mutations in different synthetic systems leading to loss of virulence.

SUMMARY.

The loss of mouse virulence shown by mutants of *Bact. typhosum* with requirements for purines or for *p*-aminobenzoic acid is attributable to the limited availability of these specific growth factors in the host, preventing the development of such mutants to a toxic level. The full virulence shown by the parent type can be restored by the injection of specific growth factors into the host or by reversion of the mutants to independence of them. The low virulence of aspartic-less mutants is probably attributable to a similar cause.

The virulence of PABA-less, but not of purine- or aspartic-less mutants can be greatly enhanced by incorporating small quantities of specific growth factor in the diet of test animals. A variation in the virulence of mutants of *Bact. typhosum* experienced over several months is considered to reflect a seasonal or other variation in the availability of nutrilites in the host.

Mutants of low virulence exert a synergistic effect when injected as a mixed suspension into mice, provided such mutants have different growth factor requirements. Synergism probably results from the reciprocal excretion of specific growth factors by each type.

The low virulence of two anomalous strains having morphological, colonial or antigenic variation in addition to growth factor requirements was not attributable to their biochemical mutation.

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